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INCREASES IN TYROSINE PHOSPHORYLATION ARE DETECTABLE BEFORE PHOSPHOLIPASE C ACTIVATION AFTER T CELL RECEPTOR STIMULATION[1]

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Antiphosphotyrosine immunoblots were used to characterize tyrosine phosphorylated proteins after stimulation of the human TCR. Increased tyrosine phosphorylation was evident on at least 12 substrates within 2 min after ligation of the TCR with mAb. Analysis of the time course for increased tyrosine phosphorylation revealed distinct patterns. Increased phosphorylation of 135-kDa and 100-kDa substrates was evident within 5 s, whereas increased phosphorylation of the TCR-ζ-chain required several minutes after treatment with anti-CD3 mAb. This rapid cellular tyrosine phosphorylation occurred independent of the cell cycle, as it occurred after stimulation of resting T cells, T cell blasts, and the Jurkat T cell leukemia line. When the TCR complex was cross-linked together with the CD4 receptor by heteroconjugate anti-CD3/CD4 mAb, an increased magnitude of tyrosine phosphorylation occurred, although no new substrates could be detected. The increased tyrosine phosphorylation of the 135-kDa and 100-kDa substrates was specific in that anti-HLA class I, anti-CD6, anti-CD7, and anti-CD28 antibodies did not cause increased tyrosine phosphorylation. Anti-CD4 stimulation of resting T cells did not cause increased tyrosine phosphorylation of pp100 and pp135, suggesting that the CD4-associated kinase, *lck*, does not account for the tyrosine phosphorylation observed after TCR stimulation. Similarly, pharmacologic treatment of cells with phorbol ester and calcium ionophore did not cause increased tyrosine phosphorylation of these substrates, indicating that activation of protein kinase C or phospholipase C does not account for these early increases in tyrosine phosphorylation. The time of onset of pp100 phosphorylation, and the magnitude of phosphorylation correlated with the magnitude of calcium mobilization when cells were stimulated with different forms of TCR stimulation. When cells were labeled with [^{3}H]myoinositol and analyzed after stimulation by anti-CD3 mAb, increased tyrosine phosphorylation of the 135-kDa and 100-kDa substrates preceded the activation of phospholipase C, as measured by the appearance of inositol 1,4,5-trisphosphate. This occurred in both T cell blasts and in the Jurkat T cell line. Thus, these findings show that increased tyrosine phosphorylation is the earliest yet detected signal observed after ligation of the TCR complex, and furthermore suggest that tyrosine phosphorylation might link the TCR to the phosphatidylinositolbisphosphate hydrolysis signaling pathway.

Ag or antireceptor engagement of the multicomponent TCR activates multiple signal transduction pathways. The earliest defined event that has been previously shown to result from Ag receptor engagement is the activation of phosphoinositide-specific phospholipase C (reviewed in Refs. 1 and 2) with the generation of inositol 1,4,5-trisphosphate and diacylglycerol (3, 4). Elevation of these metabolites leads to an increase in cellular ionized calcium concentration ($[Ca^{2+}]_i$)[3] and activation of PKC, respectively (5–7). PKC activation leads to phosphorylation of many intracellular substrates, including the TCR-CD3 γ-chain (8, 9). Receptor activation by Ag, lectins, or anti-TCR mAb has also been shown to cause rapid stimulation of PTK activity, first demonstrated by observing TCR-ζ chain tyrosine phosphorylation (10–14). T cells express several PTK, including *c-src*, *lck*, and *c-fyn*. TCR stimulation of T cells results in serine phosphorylation of *c-src* (15). Considerable interest has developed regarding Ag-receptor activation of tyrosine kinase activity, because there is substantial evidence to implicate tyrosine kinases in the control of mitogenesis, cell cycle regulation and cellular transformation (16–19). It is likely that the TCR complex is an example of a receptor that is functionally coupled to a nonreceptor tyrosine kinase, because the deduced amino acid sequences of its component subunits do not contain homology with known kinases (1).

The finding that the TCR is coupled to multiple signaling pathways raises the question of whether these pathways are activated independently, in parallel. Alternatively, it is possible that ligation of the TCR results in one primary signal, which generates multiple consequent bio-

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[3] Abbreviations within this paper: APT, antiphosphotyrosine; $[Ca^{2+}]_i$, intracellular ionized calcium concentration; GMBS, maleimidobutyryloxysuccinimide; PKC, protein kinase C; PTK, protein tyrosine kinase; $InsP_2$, $InsP_3$, and $InsP_4$, inositol bis-, tris-, and tetrakisphosphate; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PIP_2, phosphatidylinositolbisphoshate.

chemical events. Earlier investigation of TCR-mediated signal transduction has shown that PTK activation occurs in the absence of PKC in cells depleted of that enzyme by prolonged, high dose phorbol ester treatment (14). Whether PTK can activate PKC could not be determined in that study. To further address these questions we have characterized the PTK substrates, and measured the kinetics of PTK activation and second messenger generation after TCR stimulation in human T cells. By using the technique of immunoblotting with APT antibodies, we find that increased tyrosine phosphorylation of a 100-kDa (pp100) and a 135-kDa cellular substrate (pp135), can occur within 5 s after stimulation of cells. The onset of increased tyrosine phosphorylation of these substrates is followed by a time lag before the onset of phospholipase C activation, thus suggesting the possibility that phospholipase C might be regulated by tyrosine phosphorylation.

MATERIALS AND METHODS

mAb. CD2 mAb 9.6 (IgG2a), CD3 mAb G19-4 (IgG1), CD4 mAb G17-2 (IgG1), CD6 mAb G3-5 (IgG1), CD7 mAb G3-7 (IgG1) CD28 mAb 9.3 (IgG2a), HLA class I mAb HIDE (IgG2a) were produced and purified as described previously (20). CD2R mAb 9-1 was a gift from Dr. Bo Dupont (21). Heteroconjugate antibodies were prepared by using GMBS and 2-imminothiolane HCl as previously described (20). Briefly, mAb A was treated with 2-imminothiolane HCl and mAb B was treated with GMBS, and the derivitized mAb were desalted and mixed together at a 1:1 molar ratio to form a stable thioether bond.

Cells. The $CD28^+$ subset of T cells was isolated from PBL by negative selection using immunoabsorption with goat anti-mouse Ig-coated magnetic particles as previously described (22). This resulted in a population of resting T cells that was ≥99% $CD3^+$, and that did not contain $CD2^+/CD3^-$ cells such as NK cells. In some instances, the CD4 subset of $CD28^+$ T cells was isolated by a subsequent negative selection procedure by coating $CD28^+$ T cells with CD8 mAb G10-1 and magnetic beads, and recovering the bead nonadherent cells by magnetic separation. These cells were >99% $CD2^+$ and >96% $CD4^+$ as determined by flow cytometry. The Jurkat T leukemia cell line E6-1 was obtained from Dr. A. Weiss and maintained in RPMI containing 10% FCS (HyClone, Logan UT) and gentamicin.

Measurement of $[Ca^{2+}]_i$ in single cells by flow cytometry. Our procedure for the measurement of $[Ca^{2+}]_i$ in single cells has been described in detail elsewhere (23). The technique permits the simultaneous measurement of $[Ca^{2+}]_i$ and cell surface antigens in large numbers of single cells, and is capable of detecting a calcium response in as few as 0.3% of cells analyzed. In kinetic experiments, the addition of stimulatory reagent was designated time = 0.

Phosphoinositol metabolism. The method originally described by Berridge et al. (24) as modified by Meek (25) was used for the separation of inositol polyphosphates by anion exchange chromatography with an automated FPLC system (Pharmacia, Piscataway NJ) with an HR5/5 mono Q anion exchange column linked to a continuous flow liquid scintillation counter (Flo-one β model IC, Radiomatic Instruments and Chemical Co., Tampa, FL) for quantitation of the inositol phosphates. Briefly, Jurkat cells were cultured in the presence of 10 μCi/mL [^{3}H]myoinositol (SA 20 Ci/mmol, NEN, Boston, MA) in RPMI containing 10% FCS. The cells are washed extensively in HBSS immediately before assay, and the cells (5×10^6/sample) equilibrated at 37°C in lithium-free medium. The cells were stimulated with anti-CD3, the reaction terminated, and water-soluble inositol phosphates separated from the polyphosphoinositides by the addition of ice-cold perchloric acid. The extraction efficiency of inositol phosphates was improved by the addition of nonlabeled Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 as described elsewhere (26). The binary buffer system in which the inositol phosphates are separated consists of buffer A, 10 mM HEPES, 0.1 mM EDTA, 0.1 mM $ZnSO_4$ (pH 7.4), and buffer B which is buffer A supplemented with 0.5 M Na_2SO_4 (25). The inositol phosphates were separated by a step gradient in which $InsP_1$ elutes at 15 to 17 min in 0.3% B, $InsP_2$ at 25.5 min in 14% B, Ins(1,3,4)P_3 at 30 min in 30% B, Ins(1,4,5)P_3 at 34.5 min in 32% B and Ins(1,3,4,5)P_4 at 41 min, 34.5% B.

Cell solubilization, electrophoresis, and Western blotting. Details of the immunoblot assay with APT antibodies have been described elsewhere (27). Cells were suspended in HBSS containing 10% serum-free complete medium (X-vivo, 10, Whittaker Bioproducts, Walkersville, MD) and activated. Stimulation was terminated by the addition of 10× lysis buffer, yielding a final concentration of 0.5% Triton X-100 containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, 25 μg/ml *p*-nitrophenylguanidinobenzoate, 10 mM iodoacetamide, and phosphatase inhibitors (1 mM sodium orthovanadate, and 5 mM EDTA). After lysis at 4°C, the nuclei were pelleted and the supernatants subjected to SDS-PAGE on a 7.5% or 12.5% gel as previously described (27). Proteins were transferred to 0.22 μM nitrocellulose and the filters probed with affinity purified APT antibodies as previously described (14, 27, 28). These antibodies have been repeatedly shown to be specific for phosphotyrosine. The filters were labeled with ^{125}I-staphylococcal protein A (ICN, Irvine, CA) and exposed to x-ray film. Autoradiographs were scanned with a Pharmacia LKB Biotechnology Inc. Ultroscan SL laser densitometer.

Immunoprecipitation. Because TCR-ζ was not detected on immunoblots of 7.5% gels, TCR-ζ was analyzed by immunoprecipitation with rabbit anti-ζ antiserum, elution in Laemmli sample buffer, transfer to nitrocellulose, and immunoblot analysis of tyrosine phosphorylation as described elsewhere (12, 29).

Reagents. PMA was from LC Services Corp (Woburn, MA). Indo-1-AM was from Molecular Probes (Junction City, OR). Ionomycin was from Calbiochem (San Diego, CA). Rabbit antiserum to phosphotyrosine was prepared by immunizing with autophosphorylated v-*abl* kinase (28). Antibodies were purified from serum by ammonium sulfate precipitation followed by affinity purification over a phosphotyramine-agarose column.

RESULTS

Analysis of tyrosine phosphorylation after anti-CD3 stimulation. In a recent study we have characterized the tyrosine-phosphorylated substrates in murine T cell hybridoma cells after antigen or antireceptor mAb stimulation (27). TCR ζ, and two additional substrates, pp53 and pp62, were found to be tyrosine phosphorylated after stimulation as measured by immunoblotting with APT antibodies. TCR-CD3 γ-chain serine phosphorylation (8, 9, 11) and TCR-ζ chain tyrosine phosphorylation occur simultaneously, with a $t_{1/2}$ maximal of ~15 min. The tyrosine phosphorylation of pp53 and pp62, in contrast, was shown to be more rapid ($t_{1/2}$ ~2 to 3 min).

To determine the cellular substrates for the tyrosine kinase pathways in human T cells, highly purified populations of resting peripheral blood T cells were prepared and stimulated with anti-CD3 mAb for 50 s (Fig. 1). The cells were solubilized, and postnuclear supernatants were electrophoresed on SDS-polyacrylamide gels. Phosphotyrosine-containing proteins were detected after transfer to nitrocellulose and immunoblotting with affinity-purified APT antibodies and ^{125}I-labeled protein A. Marked increases in the phosphorylation of at least seven substrates were detected within 1 minute after stimulation of resting T cells.

The increased tyrosine phosphorylation that occurred in resting T cells was specific to CD3 activation (Fig. 2). Cells stimulated with CD4 or CD28 antibodies (Fig. 2) or CD6 or CD7 antibodies (not shown) did not demonstrate increased tyrosine phosphorylation. This was true whether cells were stimulated with homodimeric conjugate mAb preparations (Fig. 2) or with native antibody (not shown). Furthermore, the failure to observe increased tyrosine phosphorylation after stimulation via these other cell surface molecules was not likely to represent suboptimal cross-linking, as cells stimulated with biotin-conjugated anti-CD4, anti-CD28, and anti-HLA class I antibodies and cross-linked with avidin did not demonstrate increased tyrosine phosphorylation (not shown).

The Jurkat T leukemia cell line is often used as a model to study Ag activation of mature T cells. More proteins

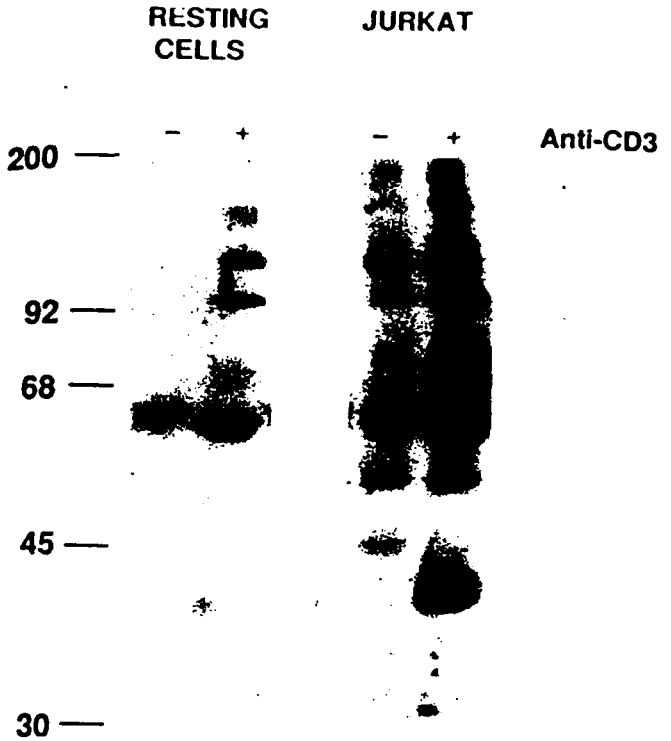

Figure 1. Effects of CD3 stimulation on cellular phosphorylation. Resting T cells (10×10^6/ml) or Jurkat cells (3×10^6/ml) were suspended in HBSS containing 10% serum-free complete medium. The cells were stimulated by addition of CD3 mAb G19-4 (10 μg) and lysed after 50 s (T cells) or 90 s (Jurkat cells). Postnuclear supernatants (1×10^8 cell equivalents [T cells] and 3×10^5 cell equivalents [Jurkat]) were electrophoresed, the proteins transferred to nitrocellulose, and APT immunoblotting performed as described in *Materials and Methods*. Autoradiography was for 1.5 days. Phosphorylated TCR-ζ is not shown, as it migrates as a 21-kDa substrate (12).

exhibited constitutive tyrosine phosphorylation in the Jurkat cells (Fig. 1); however, at least 12 proteins showed increased tyrosine phosphorylation within 90 s after stimulation by anti-CD3. There is a high degree of overlap in phosphorylated substrates in the resting T cells and the Jurkat line, though the intensity of substrates in the Jurkat on a per cell basis is clearly greater. Similar results were also obtained using a different affinity purified APT antibody for blotting, prepared by immunizing rabbits with phosphotyrosine-coupled keyhole limpet hemocyanin, rather than with v-*abl* (data not shown).

T cell blasts, prepared by culture in the presence of plastic-adsorbed CD3 antibody and IL-2 for 1 wk, were also examined for increased tyrosine phosphorylation. Immunoblot analysis of postnuclear supernatants from the T cell blasts was similar to that of Jurkat cells, in that there was increased constitutive tyrosine phosphorylation of several substrates between 55 kDa and 75 kDa (Fig. 3*A*). After stimulation with anti-CD3, there was increased phosphorylation of several substrates, although the magnitude of the signal was less than that seen in resting T cells. For reasons that become clear below, we have focused our initial studies on two of the substrates, pp135 and pp100. These substrates show increases in phosphorylation in primary resting T cells, T blasts, and Jurkat cells after TCR stimulation.

Specificity of tyrosine phosphorylation after activation with mAb conjugates. The next series of experiments were carried out to further characterize the specificity of activation. A panel of chemically cross-linked mAb homo- and heteroconjugate preparations were used to stimulate cells. These reagents have well defined effects on phospholipase C activation and on T cell proliferation (20, 30). In this experiment (Fig. 3*A*), anti-CD3 stimulation had a minimal effect on tyrosine phosphorylation, perhaps due in this case to the mAb (OKT3) used, because the anti-CD3 mAb G19-4 was a potent stimulator of tyrosine phosphorylation (Fig. 3*B*). However, increased cross-linking of CD3, as provided by the addition of a homoconjugate OKT3/OKT3 preparation, resulted in increased tyrosine phosphorylation compared with cells stimulated with OKT3 native antibody (Fig. 3*A*). Cross-linking of CD4, as provided by a homoconjugate CD4/CD4 antibody, did not cause increased tyrosine phosphorylation over a variety of time points (Figs. 2 and 3*A*). In contrast, there was a strong augmentation of tyrosine

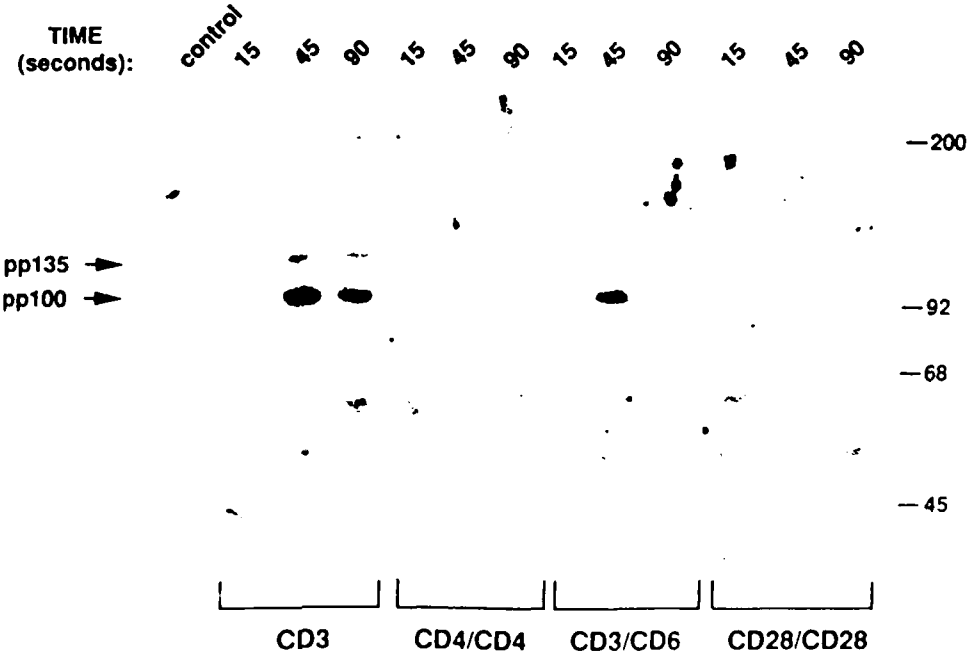

Figure 2. TCR specificity to trigger increased tyrosine phosphorylation. Resting T cells (8×10^6/sample) were stimulated by addition of CD3 mAb G19-4, CD3/CD6 heteroconjugate mAb, or homoconjugate CD4/CD4 G17-2 mAb or CD28/CD28 mAb preparations (10 μg), and stimulation terminated at the indicated time by the addition of ice-cold lysis buffer containing phosphatase inhibitors. Postnuclear supernatants (3×10^6 cell equivalents) were analyzed by immunoblot analysis for PTK activity as described in Figure 1. Autoradiography was for 2 days.

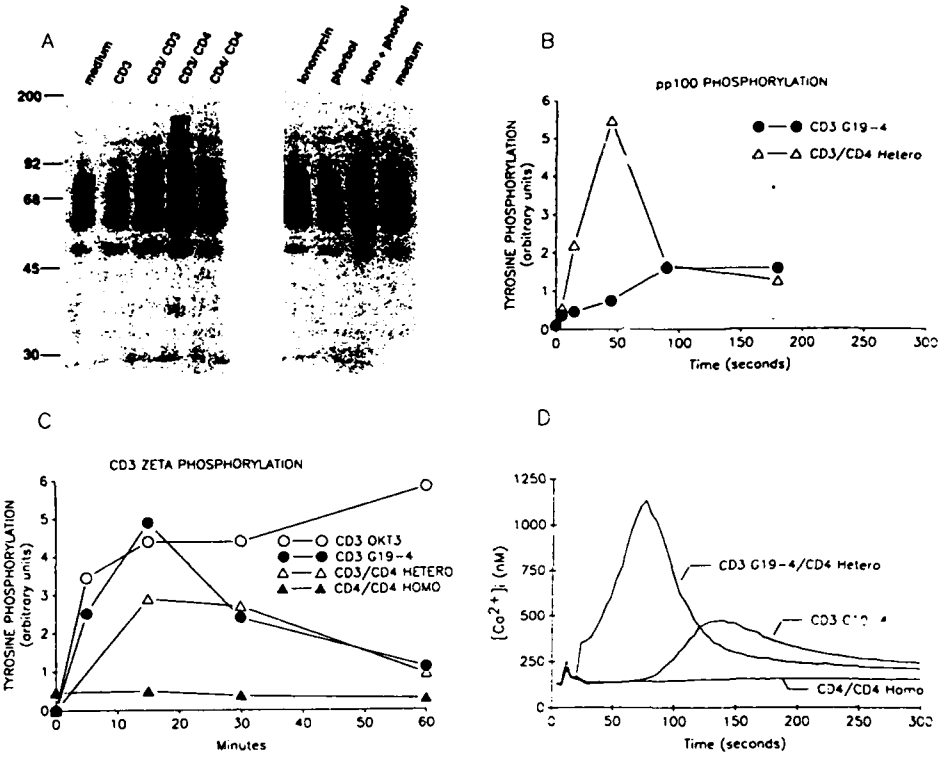

Figure 3. (*A*) CD3-receptor associated tyrosine phosphorylation is augmented by CD4 and is not mimicked by calcium ionophore or phorbol ester. $CD4^+$ T cell blasts were prepared by culture on plastic-adsorbed CD3 mAb for 2 days and in IL-2 for a further 5 days. Cells were stimulated with CD3 OKT3, CD3 OKT3/OKT3 homoconjugate, CD3/CD4 heteroconjugate, or CD4/CD4 homoconjugate mAbs at 10 μg/ml and lysed after a 5-min incubation. Alternatively, the cells were cultured for 5 min in ionomycin 0.1 μg/ml or PMA 3 ng/ml and APT immunoblotting performed as described in Figure 1. Autoradiography was for 12 h (*B–D*). Kinetics of pp100 and CD3 ζ tyrosine phosphorylation, and calcium mobilization. $CD4^+$ resting T cells were stimulated with CD3 mAb (native, homoconjugate or CD3-CD4 heteroconjugate preparations, 10 μg/sample) and solubilized after the indicated incubation. *B*, Electrophoresis and immunoblotting were performed as in Figure 1 for analysis of pp100, whereas CD3 ζ phosphorylation was detected by immunoprecipitation (*C*). The kinetics of phosphorylation were determined by densitometry of autoradiograms. *D*, The time course of calcium mobilization was analyzed by loading cells with Indo-1 and measuring the mean $[Ca^{2+}]_i$ by flow cytometry.

phosphorylation after cells were stimulated with an anti-CD3/CD4 heteroconjugate antibody preparation. The ability of CD4 to potentiate the tyrosine phosphorylation in association with CD3 stimulation was specific; heterooconjugate CD3/CD6 (Fig. 2) and CD3/CD7 (not shown) antibody preparations did not cause increased tyrosine phosphorylation above that of CD3 alone. Therefore, the hierarchy of potency of tyrosine phosphorylation after mAb stimulation: anti-CD3/4 > anti-CD3/3 > anti-CD3 ≫ anti-CD4, is similar to that seen in functional studies (30) and in calcium flux measurements (20, 30) (see below).

The CD3-stimulated tyrosine kinase activity was not the result of increased cytoplasmic calcium concentration or the activation of PKC, as stimulation by the calcium ionophore ionomycin, and the phorbol ester PMA did not cause a reproducible increase in tyrosine phosphorylation at the time points examined (Fig. 3*A*). Thus, these findings are similar to previous reports of a murine T cell hybridoma line where Ag-receptor associated tyrosine kinase activity was not attributable to rises in $[Ca^{2+}]_i$ or PKC activation (14, 27).

Time course of phosphorylation after TCR and CD4 stimulation. We next studied the kinetics of substrate tyrosine phosphorylation. We focused on cellular substrates that exhibited the earliest onset of increased tyrosine phosphorylation after CD3 stimulation, based on the notion that these substrates would likely represent primary signaling events closely coupled to the binding of Ag to the TCR. The earliest substrates to exhibit increased tyrosine phosphorylation after CD3 stimulation are pp135 and pp100. The phosphorylation of these substrates is even more rapid than that seen in the murine system (27); phosphorylation of pp135 is half-maximal at ~25 s after stimulation, whereas that of pp100 is half-maximal ~30 s after stimulation by an optimal amount of CD3 G19-4 mAb (Figs. 3*B* and 4). The magnitude of phosphorylation could be increased and kinetics of phosphorylation accelerated when cells were stimulated with anti-CD3/CD4 heteroconjugate antibodies. The rapidity of pp135 and pp100 phosphorylation can be contrasted to the kinetics of TCR ζ chain phosphorylation. ζ Chain phosphorylation required 15 to 60 min to reach maximum (Fig. 3*C*). Moreover there is *no accentuation of* ζ chain phosphorylation by CD3/CD4 heteroconjugates, in that CD3 mAb G19-4 was equivalent to the CD3/CD4 G19-4 G17-2 heteroconjugate preparation in 3 of 4 time points examined. Why OKT3 leads to potent and prolonged ζ chain phosphorylation despite minimal phosphorylation of the other substrates is not known. In contrast, ζ chain tyrosine phosphorylation induced by G19-4 is relatively short lived. Thus qualitative and kinetic differences between ζ chain and the other substrate tyrosine phosphorylation distinguish two sets of substrates in human peripheral T cells.

The tyrosine phosphorylation experiments by using the mAb conjugates were performed in parallel with flow cytometric analysis of $[Ca^{2+}]_i$ (Fig. 3, *B–D*). As previously reported (20, 23, 31), anti-CD3 G19-4 mAb stimulation results in an initial sharp rise and then a persistent plateau in calcium levels at about 250 nM. Cross-linking of the CD4 receptor with the CD4/CD4 homoconjugate did not cause calcium mobilization (not shown) or increased tyrosine phosphorylation (Fig. 3*A*), but simultaneous cross-linking of CD3 and CD4 caused a striking augmentation and acceleration of the signal. The kinetics of calcium flux and the level of calcium rise correlates well with the level of tyrosine phosphorylation of the pp135 and pp100 substrates (Fig. 3, *B* and *D*), and not with tyrosine phosphorylation of the TCR-ζ-chain (Fig. 3*C*), as will be shown more extensively below.

Time course of pp135 and pp100 phosphorylation, increased $[Ca^{2+}]_i$, and Ins(1,4,5,)P_3 production after CD3 stimulation. The extremely rapid appearance of pp100

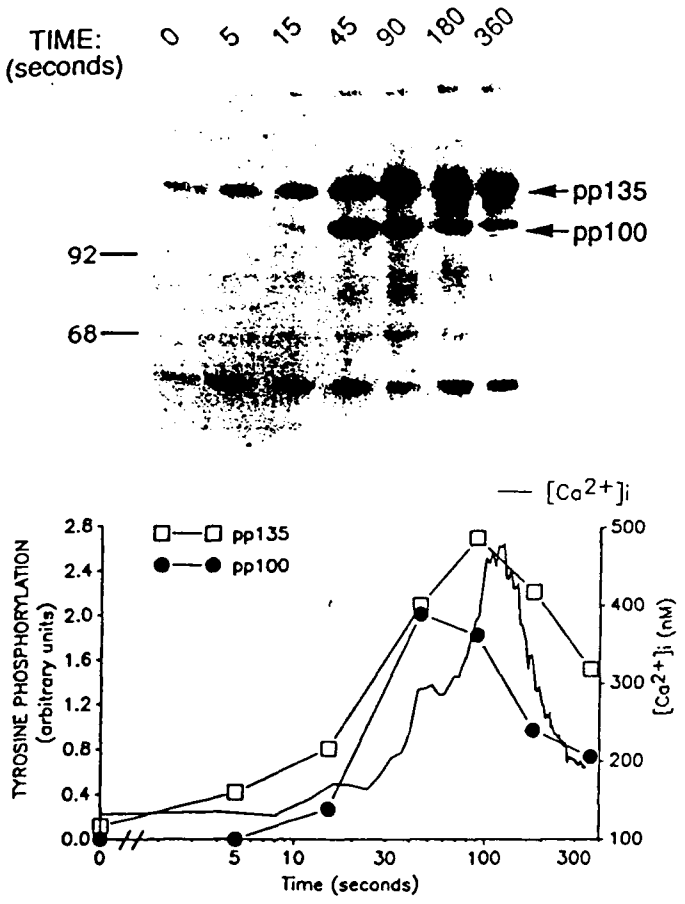

Figure 4. Kinetics of pp135 and pp100 tyrosine phosphorylation and calcium mobilization after CD3 stimulation. Resting T cells were loaded with Indo-1 and stimulated with CD3 mAb G19-4 (10 μg) at time = 0 s. Tyrosine phosphorylation of pp135 and pp100 kDa substrates was determined by immunoblot analysis with APT antibodies as described in Figure 1. The autoradiogram shown in the top panel was exposed for 1.5 days. The data from the *top panel* were quantitated by densitometry and plotted on the left axis of the *lower panel*. Changes in mean $[Ca^{2+}]_i$ were determined by flow cytometry and are plotted on the right axis of the lower panel vs time on a logarithmic scale.

and pp135 tyrosine phosphorylation raised the question of whether TCR-activated tyrosine kinase activity might precede the activation of phospholipase C and the consequent appearance of Ins(1,4,5)P_3 and 1,2-*sn*-diacylglycerol, breakdown products of PIP_2. We were unable to measure Ins(1,4,5)P_3 production in resting T cells, due to the difficulty of achieving metabolic labeling with [^{3}H] myoinositol. Thus, we compared the onset of tyrosine phosphorylation with calcium mobilization in resting T cells, as the onset of calcium mobilization is thought to occur within msecs after the production of Ins(1,4,5)P_3. When resting T cells were loaded with Indo-1 and stimulated with anti-CD3 mAb G19-4, increased tyrosine phosphorylation of pp135 clearly preceded increased $[Ca^{2+}]_i$ (Fig. 4). Increased tyrosine phosphorylation of pp135 was first detectable within 5 s after CD3 stimulation and pp100 phosphorylation was seen at 15 s, whereas calcium mobilization required ~30 s to occur. Similarly, the time of half-maximal phosphorylation of pp135 was 25 s, whereas the time to half-maximal increase in $[Ca^{2+}]_i$ was 72 s. This experiment confirms the rapidity of tyrosine phosphorylation and suggests that it precedes the sharp rise in $[Ca^{2+}]_i$.

To further define the temporal relationship of tyrosine kinase activation with phospholipase C activation after TCR stimulation, we next carried out experiments to stimulate cells with different CD3 mAb that cause different temporal patterns of calcium mobilization. In previous studies we found that stimulation of T cells with CD3 mAb G19-4 causes increased $[Ca^{2+}]_i$ with a characteristic delayed pattern when compared to CD3 stimulation by mAb 38.1 (31). The explanation for the difference in kinetics is not fully understood, but is likely related to the increased cross-linking provided by the pentameric 38.1 mAb compared with the bivalent G19-4 IgG1 mAb (32). Purified resting T cells were loaded with Indo-1 and stimulated with CD3 mAb 38.1 or with CD3 mAb G19-4 and changes in mean $[Ca^{2+}]_i$ recorded continuously for 6 min (Fig. 5 and Table I). In addition, the kinetics of pp100 and pp135 phosphorylation were assayed by immunoblotting. A suboptimal dose of G19-4 (7 μg) was chosen that results in a decreased magnitude of calcium mobilization, compared to the optimal dose used in Figure 4. The time to half-maximal increase in $[Ca^{2+}]_i$ was shorter for the cells stimulated with 38.1 mAb (50 s) than for cells stimulated with G19-4 (145 s). Densitometric analysis of the immunoblot showed that tyrosine phosphorylation of pp100 and pp135 was rapid, and half-maximal ~10 s after CD3 38.1 stimulation. As predicted, tyrosine phosphorylation of pp100 after CD3 G19-4 stimulation was much slower, and half-maximal only at ~60 s. With G19-4 stimulation (Fig. 5 and Table I), there is a low level increase in $[Ca^{2+}]_i$ between 5 and 90 s before the marked increase occurring later. The significance of this early component is not clear; it appears to parallel the increase in pp135 phosphorylation. However with either mAb, the onset of pp100 and pp135 tyrosine phosphorylation preceded the onset of the major $[Ca^{2+}]_i$ spike, and appears with both to be temporally correlated with the onset of this increased calcium level. When the magnitude of pp100 tyrosine phosphorylation was examined, it was found that the peak of pp100 tyrosine phosphorylation correlated with the form of CD3 stimulation (i.e., mAb 38.1) that resulted in the higher magnitude of calcium mobilization (251 nM vs 151 nM with G19-4) (Table I). In contrast, the magnitude of pp135 phosphorylation was not related to the magnitude of calcium mobilization; the maximal observed densitometric value for pp135, 0.81, was obtained in cells stimulated with CD3 mAb G19-4, which resulted in a lower $[Ca^{2+}]_i$ compared to mAb 38.1.

The shape of the tyrosine phosphorylation kinetic curves suggests regulation by both kinases and phosphatases. There is a rapid decrease in the level of phosphorylation of pp100 after the maximal level is reached; the effect on pp135 phosphate levels is not as acute, but is present. In one case (Fig. 5) pp100 tyrosine phosphate levels are decreasing even as calcium levels are rising. The levels of phosphate we have measured are steady state levels, and reflect both kinase and phosphatase activity. Unfortunately phosphate turnover studies are not possible with immunoblot techniques; the substrates will have to be isolated in order to further address the mechanism of regulation of TCR-stimulated substrate phosphorylation. Together, the results indicate that the onset of pp135 phosphorylation precedes calcium mobilization, and that the onset of pp100 phosphorylation precedes and is closely correlated temporally with the onset of calcium mobilization. The magnitude of calcium

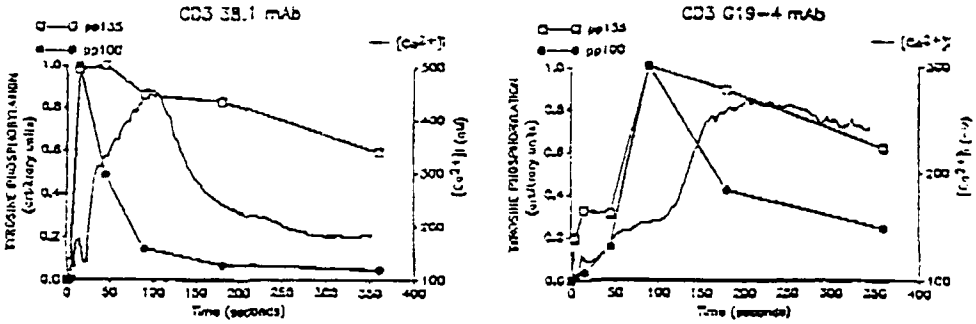

Figure 5. Temporal relation of pp135 and pp100 tyrosine phosphorylation and calcium mobilization after differing modes of CD3 stimulation. Resting T cells were loaded with Indo-1 and CD3 mAbs 38.1 (10 μg) or G19-4 (7 μg) added at time = 0. The time course of tyrosine phosphorylation of pp135 and pp100 was determined by densitometric analysis of immunoblots, and changes in mean $[Ca^{2+}]_i$ determined by flow cytometry as described earlier. The densitometric values were normalized to 1.0 to facilitate temporal comparison; the absolute values are shown in Table I.

TABLE I

Effects of CD3 stimulation on tyrosine phosphorylation of pp100 and pp135[a]

Time (s)	CD3 mAb 38.1			CD3 mAb G19-4		
	$[Ca^{2+}]_i$ (nM)[b]	pp100 (a.u.)[c]	pp135 (a.u.)	$[Ca^{2+}]_i$ (nM)	pp100 (a.u.)	pp135 (a.u.)
0	0	0.00	0.00	0	0.00	0.00
5	5	0.07	0.04	17	0.06	0.16
15	180	7.38	0.46	12	0.12	0.26
45	252	3.57	0.47	36	0.53	0.25
90	290	1.06	0.41	49	3.46	0.81
180	92	0.51	0.39	151	1.44	0.72
360	32	0.36	0.28	133	0.83	0.50

[a] Resting T cells were loaded with Indo-1 and stimulated with CD3 mAb 38.1 and G19-4. The absolute values for tyrosine phosphorylation of pp100 and pp135, and the mean $[Ca^{2+}]_i$ above base line are shown for the experiment depicted graphically in Figure 5.

[b] Incremental value (in nM) of $[Ca^{2+}]_i$ above base line of 131 nM.

[c] a.u., arbitrary units.

mobilization appears more correlated with the magnitude of pp100 phosphorylation. Furthermore, the complexity of the curves for tyrosine phosphorylation and calcium mobilization indicates that there are multiple controls on these events.

Previous studies have shown that the time course of appearance of $InsP_3$ is rapid, with the onset of detectable $InsP_3$ at ~30 s after CD3 stimulation of the Jurkat cell line (3, 4). We used FPLC and an elution system that allows the separation of Ins(1,4,5)P_3 from Ins(1,3,4)P_3 to measure phospholipase C activation, and immunoblotting to measure tyrosine kinase activation in Jurkat cells. Jurkat cells were loaded with [^{3}H]myoinositol and stimulated with CD3 mAb in lithium-free medium. Water soluble inositol phosphates were measured after lysis of cells with perchloric acid, or cells were solubilized in lysis buffer containing Triton X-100, EDTA and sodium orthovanadate, and Western blot analysis performed with APT antibody (Fig. 6). The time to half-maximal Ins(1,4,5)P_3 concentration was 29 seconds, and thus, even earlier than previous reports that measured the net concentration of Ins(1,4,5)P_3 and Ins(1,3,4)P_3 (3, 4). The time to half-maximal pp135 and pp100 phosphorylation was 14 s and 20 s, respectively. As can be seen in Figure 6, increased tyrosine phosphorylation of pp135 was clearly detectable before the onset of Ins(1,4,5)P_3 production. Similar results have also been obtained with T cell blasts, with the onset of increased tyrosine phosphorylation preceding the onset of Ins(1,4,5)P_3 production (not shown). In contrast, the time to half-maximal phosphorylation of pp100 is slower ($t_{1/2}$ = 20 s), and appears coincident with Ins(1,4,5)P_3 production. Together, the above results show that increased tyrosine phosphorylation of cellular substrates occurs before phospholipase C activation in resting and activated T cells, and in the Jurkat line.

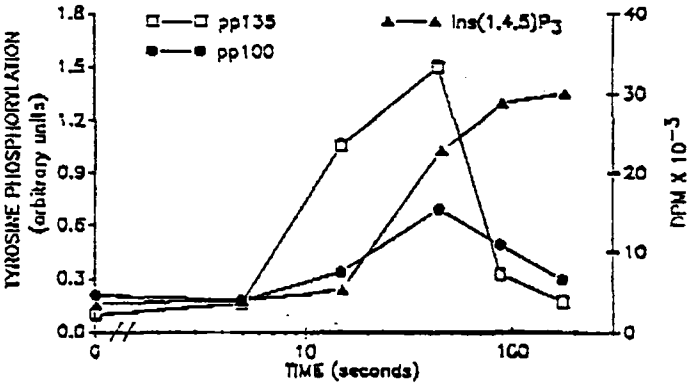

Figure 6. Kinetics of tyrosine phosphorylation and Ins(1,4,5)P_3 production after CD3 stimulation. Jurkat cells were loaded with [^{3}H]myoinositol and stimulated with CD3 mAb G19-4 10 at time = 0. The time course of pp135 and pp100 kDa substrate phosphorylation were determined by immunoblot analysis and the results of densitometric analysis depicted on the left axis. The time course of Ins(1,4,5)P_3 concentration was determined by isolation of inositol phosphates by FPLC and the results quantitated as dpm by using an in-line scintillation counter as described in *Materials and Methods*.

DISCUSSION

The characterization of cellular substrates of tyrosine kinase pathways has been made possible by the development of APT antibodies. These reagents are specific for phosphotyrosine as demonstrated by the inhibition of immunoblotting with phosphotyrosine and phenylphosphate but not with phosphoserine or phosphothreonine (27, 28). Using these reagents we have identified a number of rapidly ($t_{1/2}$ 2 to 3 min) tyrosine phosphorylated proteins in murine T cell hybridomas (27). In this study even more rapid phosphorylation (onset ≤5 s) has been identified in human peripheral resting and activated T cells, and in a human T cell line, Jurkat. There is some variability in the relative magnitude of phosphorylation of these substrates (Figs. 1 and 2); this most likely rep-

resents experimental variation in cell donors and/or reagents. In all cases, enhanced tyrosine phosphorylation is seen only after stimulation with reagents that are capable of activating T cells, such as Ag, antireceptor mAb, and in murine cells, anti-Thy-1 mAb. Pharmacologic elevation of $[Ca^{2+}]_i$ by ionophore or activation of PKC by PMA also fails to cause increased tyrosine phosphorylation of these early (pp100, pp135) substrates, indicating that the PTK activation does not follow Ca^{2+}-dependent kinase or PKC activation, or activation of phospholipase C (Fig. 3).

Antibody-mediated cross-linking of the CD4 molecule has been shown to activate the associated T cell tyrosine kinase, *lck* (33, 34), and lead to TCR-ζ chain tyrosine phosphorylation (35). The conditions used in the current study to cross-link the CD4 molecule, i.e., anti-CD4 mAb homoconjugates do not result in increased $[Ca^{2+}]_i$, substrate tyrosine phosphorylation (Fig. 3), or T cell activation of mature T cells (data not shown). In contrast, in other studies of thymocytes and of some leukemic cell lines, CD4 cross-linking itself is able to cause increased substrate phosphorylation (J. A. Ledbetter, unpublished data). Since TCR cross-linking results in a characteristic pattern of substrate phosphorylation and T cell activation, the absence of these effects with CD4 cross-linking suggests that the CD4 molecule and its associated kinase, *lck*, are not in the direct pathway of TCR-mediated activation in mature T cells. It is possible, however, that CD4 and the TCR are associated on the surface of cells, and that TCR stimulation alone results in *lck* activation. Finally, CD4-*lck* may have a role in modulating or amplifying the TCR-associated tyrosine kinase pathway. This view is supported by our observation that CD3/CD4 cross-linking had a marked effect on both the kinetics and amplitude of the tyrosine phosphorylation response (Fig. 3). Perhaps this reflects the role of CD4-*lck* during TCR interaction with MHC and Ag when CD4 can also engage MHC molecules.

We are not aware of previous studies of T cells that have characterized in detail the onset of phospholipase C activation. In both Jurkat cells (Fig. 6) and in T cells blasts (data not shown), we find no increase in water soluble inositol phosphates for the first 15 s after cellular activation by CD3 antibodies; this is followed by a sudden increase in Ins(1,4,5)P_3 that is near maximal at 45 s. We used FPLC and an elution system that allows the separation of Ins(1,4,5)P_3 from Ins(1,3,4)P_3. This was done because Ins(1,3,4)P_3 is generated by the conversion of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4, and then to Ins(1,3,4)P_3 (36). Previous studies using Dowex chromatography did not allow the resolution of the $InsP_3$ isomers. It was thus formally possible that the kinetics of the production of $InsP_3$ were actually more rapid than reported, due to the contamination with the more slowly produced Ins(1,3,4)P_3 component. The large magnitude of the signal detected in our inositol phosphate chromatography system, together with the sudden onset of the signal and the fact that analysis of cells by flow cytometry at the single cell level reveals a homogenous onset of calcium response (data not shown), leads us to conclude that we have accurately measured the onset of phospholipase C activation.

The finding that increased tyrosine phosphorylation precedes phospholipase C activation, and that pp100 phosphorylation correlates with $[Ca^{2+}]_i$ mobilization suggests that tyrosine phosphorylation may be a regulatory event linking the TCR to the PIP_2 hydrolysis signaling pathway. Recent evidence from other systems supports this concept. Several groups have shown that the binding of EGF to the EGF receptor, or the binding of PDGF to the PDGR receptor, receptors with protein tyrosine kinase activity, induces tyrosine phosphorylation of phospholipase C-γ (37–40). There is evidence that the EGF receptor is directly coupled to phospholipase C because antibodies to the EGF receptor could coimmunoprecipitate phospholipase C. Furthermore, a tyrosine kinase inhibitor prevented EGF-induced tyrosine phosphorylation of phospholipase C, and EGF-induced calcium mobilization. These findings from studies of growth factor receptors that encode tyrosine kinase activity, in conjunction with our results, suggest that TCR-induced cell activation may be an example where a nonreceptor-associated protein tyrosine kinase is functionally coupled to phospholipase C. As yet, we have no direct evidence that either pp135 or pp100 is phospholipase C or is associated with phospholipase C, as we have been unable to immunoprecipitate these substrates with mAb (kindly provided by S. G. Rhee) that were raised against phospholipase C isoenzymes expressed in bovine brain (41). An additional possibility would be that phospholipase C activation and calcium signaling are not closely linked. The TCR could initiate calcium increases via activation of calcium pumps or channels, and in such a case, the substrates that we have identified could represent the calcium channel or regulatory proteins associated with the channel or pump. Finally, the potential role of guanine nucleotide-binding regulatory proteins in TCR-mediated activation of phospholipase C remains to be clarified (42). Recent studies have shown that tyrosine kinase inhibitors prevent calcium mobilization after EGF but not bradykinin stimulation of HER14 cells (38), suggesting that in the same cell, phospholipase C activity may be regulated by mechanisms that involve tyrosine kinases and by a distinct mechanism. By analogy to these studies, it is possible that phospholipase C activity in T cells is regulated by more than one mechanism.

A question that remains unresolved is how the different classes of TCR that contain either ζ chain homodimers or ζ-η heterodimers that have been described in murine T cells (43) are related to our findings in human T cells. In the murine system, there is evidence that receptors that bear TCR ζ-η heterodimers activate phospholipase C, and that those lacking TCR-η activate increased tyrosine phosphorylation of the TCR-ζ chain but are not coupled to PIP_2 hydrolysis (44). The work that we have presented, and the precedents from the EGF and PDGF systems (37–40) suggest that the tyrosine kinase pathway has some role in activating the PLC pathway. The function of the ζ chain relative to both phospholipase C and tyrosine kinase activation remains to be determined.

The relationship of tyrosine phosphorylation and the biologic function of T cells remains to be defined. The current work supports the idea that this pathway is important early in activation after receptor occupancy. Other work suggests that tyrosine phosphorylation and not PIP_2 turnover regulates the differentiated function of T cells. T cell hybridomas lacking TCR-η chain and without TCR-activated phosphoinositol turnover produce nor-

mal levels of IL-2 after Ag stimulation or receptor cross-linking by antibody (45). In another series of experiments, infection of a murine T cell hybridoma with the unregulated v-*src* PTK results in constitutively elevated levels of tyrosine phosphorylation and constitutive production of IL-2 (J. J. O'Shea, personal communication). Therefore, regulation of multiple cellular functions in T cells could be controlled by tyrosine phosphorylation. Substrate characterization appears to be a way of understanding this pathway. Finally, in our work it has not been possible to distinguish increased tyrosine phosphorylation as a result of the activation of PTK or as a result of the inhibition of cellular protein tyrosine phosphatases. Similarly, the observed decrease in tyrosine phosphorylation reflects regulation by tyrosine phosphatases. These issues will require further investigation in view of the recent work demonstrating the presence of protein tyrosine phosphatases in lymphocytes (46, 47), and the potential role of protein tyrosine phosphatases in cellular signaling (48–50).

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